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14. ABSTRACT (200 words of the most significant findings and erase this text)
We have in the first funding year successfully generated the mouse strains required for the proposed experiments. In particular, we have generated FMRP mutant mice that also harbor the TauEGFP allele which allows the identification and purification of induced neuronal (iN) cells. We have also successfully established mouse embryonic fibroblast and tail-tip derived fibroblast cultures from these mice. Moreover, we successfully generated iN cells from these mice and characterized them molecularly. They are also being analyzed electrophysiologically in the Chen lab (see her report). Moreover, we have made very good progress in converting also human fibroblasts into iN cells. Systematic screening of additional transcription factors has revealed that the addition of NeuroD1 greatly enhances the efficiency to convert human fibroblasts into iN cells and in this condition we were also able to generate cells that are synaptically competent. However, the efficiencies are still much lower than using mouse cells and we are currently trying to improve these efficiencies.

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Project Award: Testing synaptic functions in neurons derived from Fragile-X patients

Introduction

Fragile-X syndrome (FXS) is a common form of genetic neuropsychiatric disease affecting predominantly male children. It is characterized by mental retardation, low intelligence and other cognitive abnormalities such as sensational, emotional, and behavioral problems. Most of what we have learned so far about the molecular and cellular mechansims of the disease is based on mouse models in which the disease-causing gene Fragile X Mental Retardation 1 (FMR1) has been deleted. We have recently found a method to generate neurons from skin fibroblasts in the mouse and more recently in human. We termed these cells induced neuronal (iN) cells because they shared all principal functional and biochemical features of bona fide neurons. We have generated iN cells from various somatic cells including mouse hepatocytes and more importantly also from human skin fibroblasts. The main hypothesis of this proposal is that these iN cells can be used to study molecular features of FXS in both the mouse model as proof of principle as well as using human cells.

Body

We perform this project in close collaboration with the Partner PI of the grant, Dr. Lu Chen. Some parts of the Specific Aims are done in her lab, while others are performed in mine. Please refer to her progress report regarding the tasks that her lab is responsible for. I will describe the progress that our group has made under each Specific Aim in the following:

Specific Aim 1. Characterization of synaptic properties of iN cells derived from fibroblasts of Fmr1 KO mice (Chen and Wernig lab)

Task 1. Convert fibroblasts from *Fmr1* KO mice to iN cells

We have accomplished this goal and used the same approach to convert wild type mouse fibroblasts (Vierbuchen et al., 2010 Nature). Moreover, it became clear that it is not only required to convert Fmr1 KO fibroblasts to iN cells, but we need in addition a genetic live label of iN cells. To that end we bred the Fmr1 KO mice to Tau-EGFP Knock-in mice. Tau is a neuronal-specific gene and therefore, GFP-positive cells derived from fibroblasts unambiguously labels iN cells. This allows to study these cells not only in isolation, but also in co-cultures with astrocytes (a condition known to dramatically improve synaptic maturation and synaptogenesis) or together with primary neurons, derived from the neonatal or fetal hippocampus or neocortex.

Figure 1 (next page): *Generation of iN cells from Fmr1 KO; Tau::EGFP mouse fibroblasts*. Fibroblasts were infected with the 3 transcription factors Brn2, Ascl1, Myt1l and cultures were stained 3 weeks after infection with the neuronal marker Tuj1 (blue) and the presynaptic protein V-Glut (red). The green signal stems from the Tau::EGFP reporter and indicates neuronal conversion of fibroblasts.

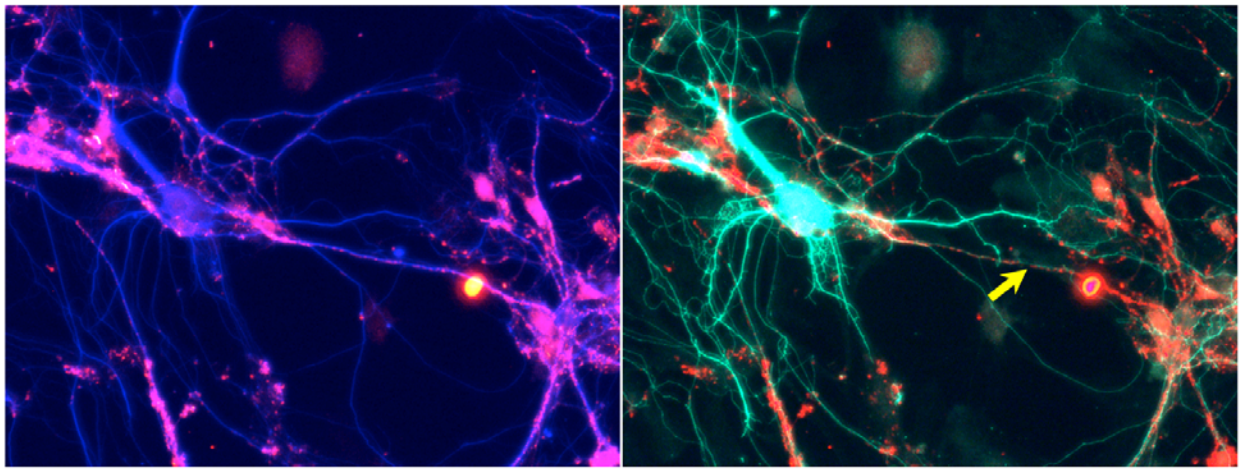
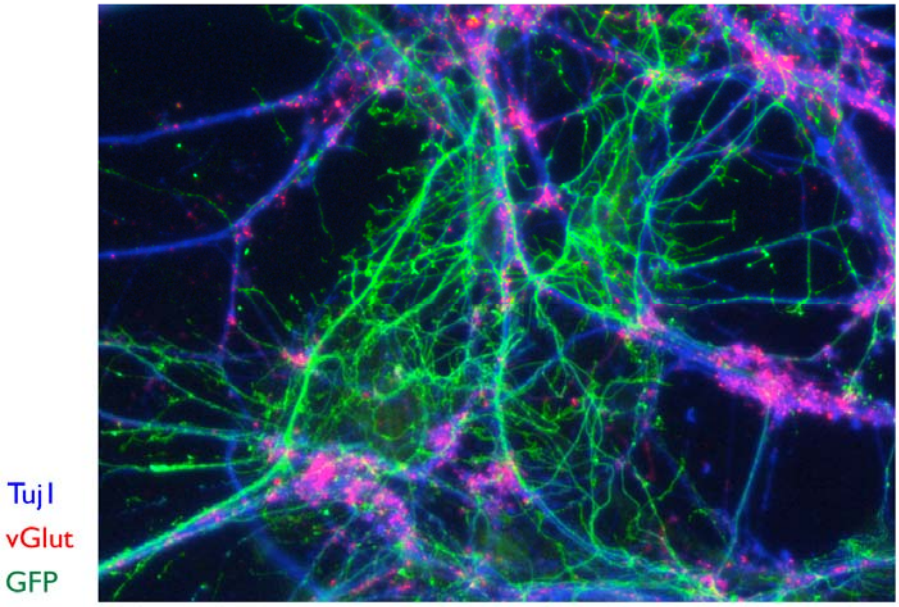


Figure 2 (below): *Successful co-culture of Fmr1 KO iN cells with primary hippocampal neurons*
Tau::EGFP-positive iN cells were sorted by FACS on day 7 after infection and plated onto a previously established culture of primary neurons derived from the neonatal rat hippocampus. Both iN cells and primary neurons are labeled with Tuj1 (blue) and are depicted with v-Glut (red). iN cells can be distinguished from the primary neurons by virtue of their EGFP label (green).



Task 2. Characterize synaptic function of iN cells derived from *Fmr1* KO mice
This task is performed by Dr. Lu Chen's lab. Please see her progress report for the progress made here.

Specific Aim 2. Generation of iN cells from FXS patients (Wernig lab)
Task 3. Derive human iN cells from human embryonic fibroblasts

We are very happy to report that we have been successful to derive iN cells from human fetal fibroblasts that are fully functional, i.e. have both active membrane properties (such as action potentials) and show synaptic input from surrounding neurons (other iN cells or primary neurons), thus are synaptically competent. We had to use an additional transcription factor: NeuroD1 to convert human cells which was not required for the mouse cells. Figure 3 shows the results of the screen that identified NeuroD1 as critical additional factor. Figure 4 shows an example of a human fetal fibroblast-derived iN cells and an example trace demonstrating synaptic input.

Figure 3: *NeuroD1 is a critical reprogramming factor to generate iN cells from human fetal fibroblasts.* We combined the BAM pool of transcription factors with several other candidate factors and found that Neurod1 consistently yielded more Tuj1-positive cells than control conditions (upper left panel). A systematic evaluation of the possible combinations of the resulting 4 factors showed that while Brn2 and Neurod1 (BN) alone is sufficient to induce a high number of Tuj1-positive cells (lower left panel), only when all four factors are combined, we see good functional responses in terms of generating cells that can elicit action potentials (right panel).

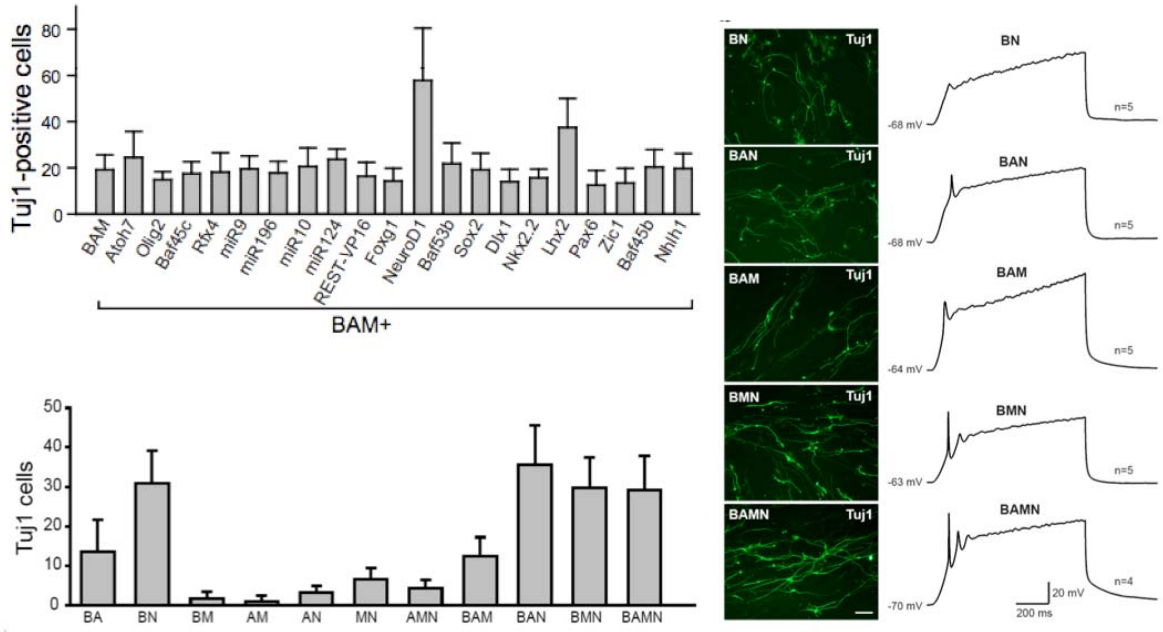
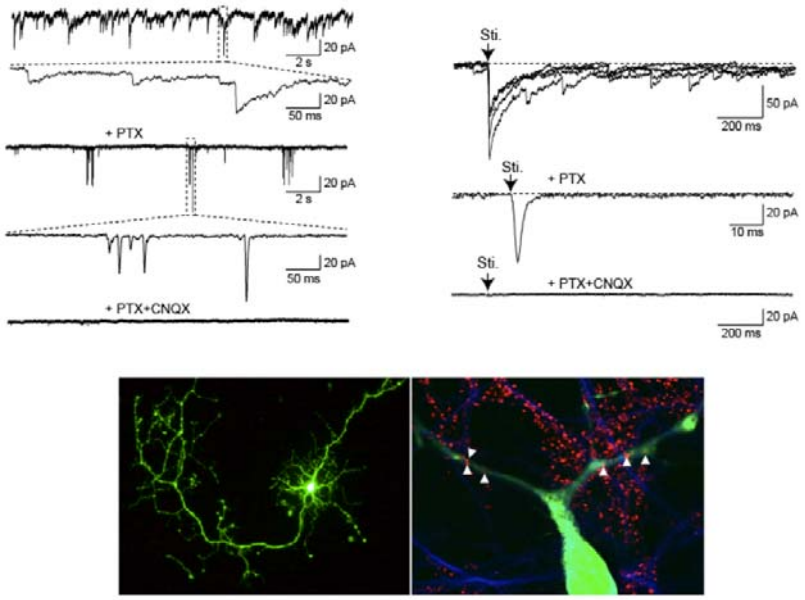


Figure 4 (next page) Human fetal fibroblast-iN cells are synaptically competent. Upper left panel shows example traces recording spontaneous synaptic events in iN cells co-cultured with primary mouse cortical neurons. Addition of Picrotoxin (PTX) blocks GABA receptors and eliminates the spontaneous inhibitory postsynaptic currents. Further addition of CNQX blocks AMPA receptor channels which mediate excitatory synaptic currents. These pharmacological characerizations demonstrate the specificity of the currents observed. The right panel shows similar synaptic currents in response to simulation (Sti.). The lower panel shows 2 example cells infected with a constitutive

GFP virus (green), co-stained with MAP2 (blue) and Synapsin (red).



Task 4. Derive human iN cells from human skin fibroblasts
We have successfully generated iN cells also from early postnatal human foreskin fibroblasts. In principle we were able to find cells with the same functional characteristics as the fetal fibroblast-derived iN cells (as shown in Figs. 3&4). However, while the efficiency of getting Tuj1-positive cells was similar, the yield of fully synaptically competent cells was much lower. Therefore, we are working on systematic ways now to improve the protocol (testing seeding densities, various media conditions, as well as small molecule inhibitors to block and activate various signaling pathways in isolation and combination).

Task 5. Derive human iN cells representing different neuronal subtypes from human skin fibroblasts (Wernig lab, months 15-24)
As planned we have not yet begun these experiments as we feel first, we need to improve the reprogramming efficiencies with human postnatal and eventually adult fibroblasts.

Task 6. Derive human iN cells from FXS patient skin fibroblasts (Wernig lab, months 18-24)
Again as planned we have not begun yet to generate iN cells directly from FXS patient fibroblasts. However, meanwhile we have developed a way to generate iN cells very rapidly from human iPS cells. When we tested the transcription factors that we use to convert fibroblasts in human iPS cells and ES cells we noticed a dramatically high conversion efficiency into iN cells. In particular NeuroD1 and Ngn2 appear to be sufficient to generate homogenous cultures of iN cells within just a few weeks. Although this is not quite the original plan, but because of this finding, this alternative route to generate iN cells became a viable back-up approach to reach the final goal of this grant.

We have therefore begun to generate iPS cells from FXS patients and test their responsiveness to the iN cell induction protocol that we have optimized for control lines. We have provided Lu Chen's lab with iN cells derived from control iPS cells for electrophysiological characterization (see her report).

Specific Aim 3. Characterization of synaptic properties in FXS-iN cells (Chen lab)
Task 7. Establish assays for synaptic function in human iN cells (Chen lab, months 6-20)
Task 8. Characterize synaptic function of human iN cells derived from FXS patient fibroblasts (Chen lab, months 20-30)

This aim is being executed in its entirety by Lu Chen's lab. Please see her report.

Specific Aim 4. Investigation of effects of mGluR1 and mAchR antagonists on synaptic properties in FXS-iN cells (Chen lab)
Task 9. Investigate effects of mGluR antagonists on synaptic functions of FXS-iN cells (Chen lab, month 31-33)
Task 10. Investigate effects of mAchR antagonists on synaptic functions of FXS-iN cells (Chen lab, month 34-36)
These aims will be investigated by Dr. Lu Chen's laboratory as planned in the future.

Key Research Accomplishments
• Generated Tau::EGFP; Fmr1 KO mice for generating iNs from fibroblasts
• Generated iN cells from fibroblasts from these mice
• First electrophysiology recordings from Fmr1 KO mouse iN cells (in collaboration with the Chen lab)
• Discovered an alternative route to generate iN cells directly from pluripotent stem cells with high efficiencies

Reportable Outcomes
We have prepared and submitted a manuscript that describes the generation of iN cells from human pluripotent stem cells. This manuscript is currently under review at Neuron.

Conclusion
Our research achievements have been so far as planned and we will continue to work on the tasks as originally proposed. We are not sure how fast we will be able to optimize the human fibroblast-iN cell conversion, but as of now everything is quite "on track". In addition, we have developed an intriguing alternative approach to generate iN cells from iPS cells that will work for sure, in case our original plan to use fibroblast-iN cells from human patients turns out to require more time to optimize.

References
Vierbuchen T, Ostermeier A, Pang ZP, Kokubu Y, Sudhof TC, Wernig M (2010) Direct conversion of fibroblasts to functional neurons by defined factors. Nature 463:1035-1041.